False negatives in screening for chromosomal abnormalities

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Helsinki 17.03.2021
Thesis
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Työn nimi - Arbetets titel - Title

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Oppiaine - Läroämne - Subject

Medicine

Työn laji - Arbetets art - Level	Aika - Datum - Month and year	Sivumäärä - Sidoantal - Number of pages
Thesis	17.03.2021	28

Tiivistelmä - Referat – Abstract

Kromosomipoikkeavuuksien ensisijainen seulontamenetelmä Suomessa on ns. varhaisraskauden yhdistelmäseulonta. Seulonnan tarkoituksena on löytää ne raskaudet, joissa riski tavallisimpiin kromosomipoikkeavuuksiin, eli 13-, 18- tai 21-trisomiaan on kohonnut.

Tavoitteet: Kun henkilö, jolla on seulottu tila, saa negatiivisen tuloksen, kutsutaan sitä vääräksi negatiiviseksi. Tämän tutkimuksen tavoite oli löytää yhteisiä tekijöitä tapauksista, joissa tulos oli väärä negatiivinen, ja verrata niitä vastaaviin tekijöihin tapauksissa, joissa tulos oli oikea positiivinen. Pyrittiin selvittämään, onko tekijöissä näiden ryhmien välillä statistisesti merkitseviä eroja.

Menetelmät: Tämä on retrospektiivinen asiakirjatutkimus, jossa tutkimusaineiston muodostavat v. 2014-2016 synnytykseen päättyneet raskaudet, joissa vastasyntyneellä on todettu joko 13- 18- tai 21-trisomia. Tässä tutkimuksessa keskityttiin vastasyntyneisiin, joilla todettiin trisomia 21. Riskilukua 1:250 käytettiin raja-arvona trisomia 21:ssä. Tutkimuksen kriteerit täyttivät 14 väärää negatiivista tapausta sekä 119 oikeaa positiivista tapausta. Analysoitavat parametrit olivat äidin ikä, raskauden kesto verikokeessa, biokemialliset markkerit PAPP-A (MoM) ja hCG (MoM), raskauden kesto ultraäänessä sekä niskaturvotus. Statistisia menetelmiä käytettiin muuttujien analysointiin.

Tulokset: Niskaturvotus selitti logistista regressionanalyysia käyttäen merkittävimmän osan vääristä negatiivisista (47,6%). Toiseksi suurin selittävä tekijä oli PAPP-A MoM (15,2%). Äidin ikä selitti 11,9% ja hCG MoM 11,1%. Raskauden kestossa verikokeessa tai ultraäänessä ei ollut ryhmien välillä merkitsevää eroa.

Johtopäätökset: Kaikki tutkitut muuttujat raskauden kestoa lukuun ottamatta selittivät statistisesti merkittävän osan vääristä negatiivisista. 14,2% vääriä negatiivisia selittävistä syistä jäivät tuntemattomiksi. Koska niskaturvotus selitti suurimman osan vääristä negatiivisista, on sen oikea-aikaiseen ja laadukkaaseen mittaamiseen panostaminen vastaisuudessa keskeistä.

Avainsanat - Nyckelord - Keywords

Trisomy, prenatal screening, false negative, combined screening, Down syndrome

Säilytyspaikka - Förvaringställe - Where deposited

Muita tietoja - Övriga uppgifter - Additional information

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Tiivistelmä - Referat – Abstract

In Finland, the primary screening method for chromosomal abnormalities is the first trimester combined screening. The aim is to find pregnancies with an increased risk for the most common chromosomal abnormalities, trisomies 13, 18 or 21.

***Objective*:** When a person with the condition screened for gets a negative result, it's referred to as a false negative. The objective of this study was to find common characteristics in false negative cases and compare them to those of true positives to see if there's a significant difference.

***Methods*:** This is a retrospective study of pregnancies between 2014 and 2016 that ended in a birth of a neonate with trisomy 13, 18 or 21. In this study we focused on the neonates with trisomy 21. A risk figure of 1:250 was used as the cut-off value for trisomy 21. There were 14 false negative cases and 119 true positive cases that qualified for our study. The parameters analyzed were maternal age, gestational age at blood tests, biochemical markers PAPP-A (MoM) and hCG (MoM), gestational age at ultrasound and nuchal translucency. They were statistically analyzed.

***Results*:** By using logistic regression, we found that the most powerful discriminating factor between false negative and true positive cases was nuchal translucency, accounting for 47.6% of false negatives. The second most powerful factor was PAPP-A MoM explaining 15.2%. Maternal age explained 11.9% and hCG MoM 11.1%.

***Conclusion*:** All the factors except for gestational age accounted for a statistically significant proportion of false negatives. The most powerful discriminating factor between false negatives and true positives was nuchal translucency. 14.2% of the discrimination remains unknown to us. Since nuchal translucency is the most powerful discriminating factor, it is essential to focus on its correct measurement.

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Abbreviations

CRL: Crown-rump length

DS: Down syndrome

FN: False negative

FNR: False negative result

FTS: First trimester screening

gws: Gestational weeks

hCG: Human chorionic gonadotropin

MoM: Multiples of the median

NIPT: non-invasive prenatal testing

NT: Nuchal translucency

PAPP-A: pregnancy-associated plasma protein A

TOP: termination of pregnancy

TP: True positive

TPR: True positive result

1 Introduction

In Finland, every expecting mother is given the opportunity to participate in prenatal screening (1). The goal of screening is to find chromosomal and structural abnormalities if present. Participating is voluntary and 84–90% of women choose to participate in prenatal screening (2,3).

Structural and chromosomal abnormalities are fairly common in fetuses and approximately three out of one hundred neonates have either a chromosomal or a structural abnormality. One third of these abnormalities is severe (4).

In Finland, the primary screening method for chromosomal abnormalities is the first trimester combined screening (FTS). It consists of blood tests (BT) and a general ultrasound (US) and has the highest sensitivity for abnormalities (4,5). The detection rate ranges between 80% and 90% when the screen positive rate is 2.5–5% (6,7).

The risk figure for a chromosomal abnormality is calculated using serum markers, maternal age, the duration of the pregnancy and fetal nuchal translucency (NT) (1). The cut-off limits of 1/250 and 1/150 are used for a positive result in screening for trisomy 21 and trisomy 18 (8). The serum markers' concentrations are affected by the mother's weight, possible gestational diabetes and smoking. The equipment used and the operator's experience affect the NT measurement (1,9).

The basic statistical measures of screening are sensitivity and specificity. The sensitivity of screening describes how well the test finds the individuals with a condition searched for, in other words, in prenatal screening a chromosomal abnormality. The specificity of the test tells how well it finds the individuals without an abnormality. By increasing the sensitivity, the specificity decreases (Figure 1) (10).

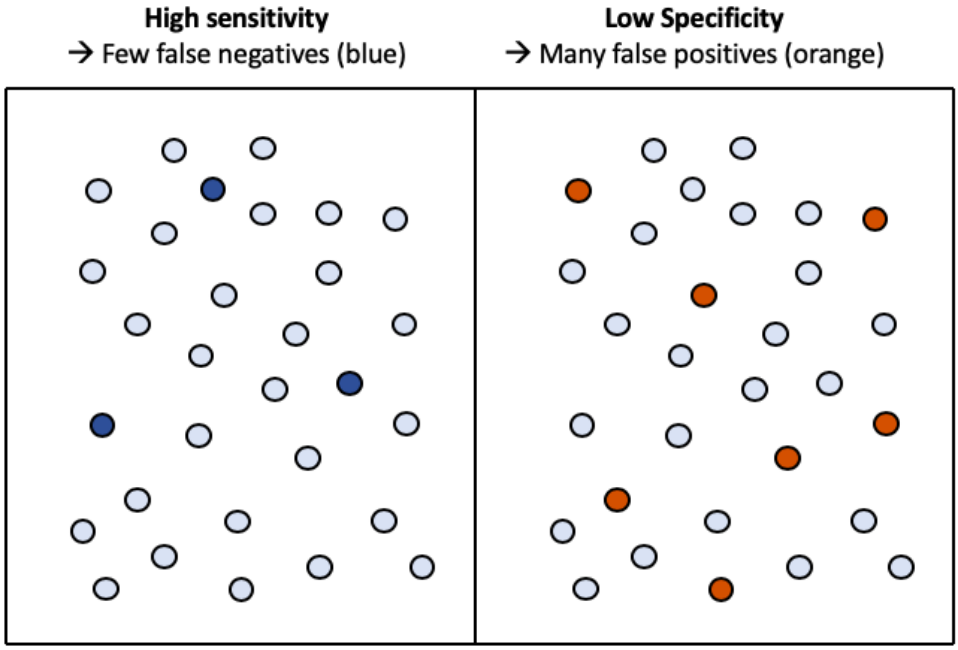

Figure 1: Illustration of the performance of a high-sensitivity, low-specificity screening. In false negatives, the screening has failed.

The aim of this study is to find out why the prenatal screening failed to find a chromosomal abnormality in the studied pregnancies. We searched for possible common factors that might have affected the sensitivity of the screening. This was done by comparing the parameters of the false negative (FN) group to the ones of the true positive (TP) group.

2 Review of the literature

2.1 Prenatal screening

Screening means systematically searching for a defect or a disease in healthy individuals (10). Screening for chromosomal abnormalities in Finland began in the 1970s. Since the risk for chromosomal abnormality increases with maternal age, at first only women between 35 and 40 years were screened using amniocentesis. Later on, a serum screening, NT measurement and finally the combination of these were used, to form FTS, which is the primary method used in Finland today (1).

2.1.1 The principles and purpose of prenatal screening

Prenatal screening is organized according to the criteria published by the World Health Organization in the 1960s and complemented by the Danish Council on Ethics (1,4). Participation is voluntary and free of charge (4,8). The purpose of prenatal screening is to find pregnancies with an increased risk for a fetal defect or a disease (11).

The goal of prenatal screening is the detection of pregnancies with chromosomal or severe structural abnormalities. Chromosomal abnormalities are mainly screened in early pregnancy and the aim is to find pregnancies with an increased risk for the most common chromosomal abnormalities, trisomies 13, 18 or 21 (5).

Prenatal screening enables the reproductive autonomy of mothers and parents and reduces both fetal morbidity and mortality (8). An early prenatal diagnosis is needed to organize the postnatal care and also offers the mother the possibility to choose termination of pregnancy (TOP) within the legal timeframe, before the end of the 24^{th} gestational week (gw) (10).

During the first outpatient appointment and before participation, prescreening information concerning the purpose and limitations of screening is given, both verbally and in writing. Informed consent is expected concerning the decision to participate or to forgo screening (8,11).

2.1.2 Organization of prenatal screening

Before the decision, the mother should be informed about all possible options of prenatal screening: to participate in a general US and screening for chromosomal abnormalities, to only participate in a general US (without screening for abnormalities) or not to participate in either of these. The organization and options for screening in Finland are presented in Figure 2. The details of the different screening methods are discussed below (8,11).

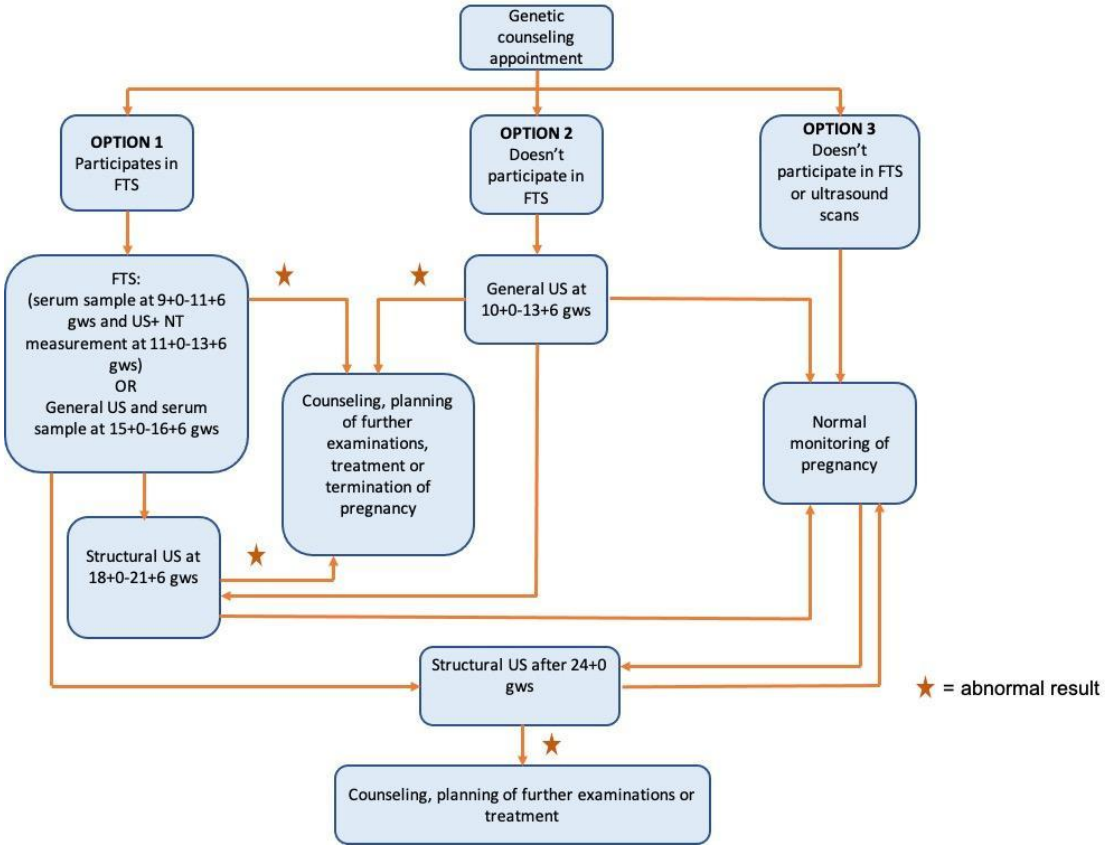

Figure 2: Organization of prenatal screening in Finland. FTS = first trimester screening, gws = gestational weeks, NT = nuchal translucency, US = ultrasound

2.1.3 Screening methods

2.1.3.1 First trimester combined screening

First trimester combined screening consists of a BT at 9+0 to 11+6 gws and a general US at 11+0 to 13+6 gws, with an NT measurement (4,10). The BT is based on measuring pregnancy-associated plasma protein-A (PAPP-A) and human chorionic gonadotropin (hCG) concentrations in the maternal serum. The values are expressed in multiples of the median (MoM) (1). The purpose of the general US is to confirm the number and heartbeat of fetuses and the gestational age using the crown-rump length (CRL) measurement. The sonographic appearance of fluid under the fetal neck area is called NT. An NT measurement of over 3 mm is a sign of an increased risk for a chromosomal abnormality (2). The US is most commonly performed by a trained midwife or a sonographer (8).

By combining the information gained from the general US and the BT, a risk figure is calculated, using maternal age, free maternal serum markers (hCG and PAPP-A) and NT measured in the first trimester general US (8,12). A value of 1:250 is used as the cut-off in trisomy 21, and 1:150 in trisomy 18, for a positive screening result and an increased risk for chromosomal abnormality. Also, an NT > 3 mm alone in the general US is considered a sign of an increased risk for chromosomal abnormality (2).

2.1.3.2 Second trimester screening

In cases of a late registration for antenatal care, incorrect dating of pregnancy or otherwise unsuccessful FTS, maternal serum can alternatively be screened in the second trimester. Alpha-fetoprotein (AFP), unconjugated estriol, ß-human chorionic gonadotropin (ß-hCG) or hCG and inhibin-A are used as markers in second trimester serum screening (1,10). The concentrations of AFP and estriol decrease in trisomy 21 and those of hCG, ß-hCG and inhibin-A increase (13). Screening methods in the second trimester are less sensitive than those in the first trimester and the DS detection rate ranges between 60% and 65% (5).

A second trimester US is performed at 18–21 gws to screen for possible structural abnormalities. All organs are examined (4,8). Possible abnormalities range from mild to life threatening and the sensitivity of the examination may depend on the severity of the abnormality (8).

Genetic counselling concerning the screening results is given to all mothers with a screen-positive result. After counselling, the opportunity to participate in further diagnostic tests, to either confirm or refute the positive screening result, is offered (4).

Instead of an invasive diagnostic test, most mothers first choose to have a non-invasive prenatal test (NIPT), which can be performed after the ninth week of pregnancy. In the NIPT, fetal extracellular DNA is extracted from the mother's blood and screened for abnormalities in chromosomes 13, 18 and 21. Sex chromosomes can also be screened. Compared to FTS, the NIPT is a more sensitive screening method, with a sensitivity of 98–100% in trisomy 21, 96% in trisomy 18 and 91% in trisomy 13. The false positive rate (FPR) is 0.09–0.13% and possible

causes include mother's tumors, vanishing twin syndrome and confined placental mosaicism. In the case of an abnormal NIPT result, an invasive test should be performed to confirm the result (2).

In the invasive test, a needle is inserted through the mother's abdominal wall and the sample is taken either from the placenta or the amniotic fluid. Chorionic villus sampling (CVS) can be used between 10 and 13 gws and amniocentesis from 14 gws onwards (2,14). Both of these tests are invasive and associated with a 0.5–1% risk of spontaneous TOP (4,5). To women over the age of 40, the NIPT or diagnostic tests can be offered directly without any preceding prenatal testing (5).

Early detection and knowledge of possible abnormalities help in planning the postnatal care. The delivery date and the method can be chosen in advance and some of the structural abnormalities can be treated with an operation after birth (10,15).

2.1.4 Aftermath

In prenatal and genetic screening, the conditions screened for can rarely be cured with conservative or operative care (11). If the screening result is positive or an abnormality is detected in the US, an appointment with a geneticist or an obstetrician is organized to explain the meaning of the result and gain information on the condition found (15).

In Finland, a fetal anomaly is legally not an acceptable cause to terminate a pregnancy after the 23rd week (4). Yearly around 50% of Down pregnancies are terminated. Women under the age of 35 terminate a third of Down pregnancies and women over 35 two thirds (14). The termination rate of pregnancies has seen a slight increase since 2006, but the proportion of terminations due to a major congenital anomaly has seen only little change (16).

2.2 Chromosomal abnormalities

At 3–8 gws, the fetus' developing organs are most vulnerable to disturbance caused by environmental factors. The risk of abnormalities also increases with increased maternal age (4). Between 2% and 3% of neonates are born with a major anomaly and around 30% of these with

a syndrome or multiple anomalies (1). In Europe, around 0.5% of neonates are born with a chromosomal abnormality and around 5.0% with a structural abnormality. Chromosomal abnormalities are often associated with structural abnormalities (8).

The chromosomal abnormality with the highest prevalence is trisomy 21, Down syndrome (DS) (8). The most common trisomies after DS are trisomies 18 and 13. In some cases, the chromosomal abnormality is restricted to only a part of the cell lines. This is called trisomy mosaicism. The clinical symptoms caused by trisomy mosaicism can be milder, but are variable and depend on the percentage of abnormal cell lines (11).

The abnormal number of chromosomes affects the fetal development. It disturbs the production of placental hormones and is ground for the measurement of placental hormones in FTS. Chromosomal abnormalities also make the connective tissue overly elastic, which might be the cause of increased NT in early pregnancy (1,8): NT > 3 mm is associated with an increased risk of chromosomal abnormalities. The thicker the NT, the greater the risk of an affected fetus (17).

2.2.1 Trisomy 21

Trisomy 21 or DS is the most common cause for developmental disability in Finland. Around 70 children are born with it each year (1). It is associated with moderate intellectual retardation and delayed growth (18). Typical clinical features of DS include a flattened facial profile, ears with an abnormal shape, a short neck and a round face. Down syndrome is sometimes associated with cardiac and/or gastrointestinal anomalies and it increases the risk of hearing disabilities, leukemia, Alzheimer's disease and hypothyreosis (11,18). Down syndrome may also predispose children to infections (11).

These features may sometimes be diagnostic, but the diagnosis of DS should always be confirmed with a chromosome analysis. The clinical features can be less distinct and the appearance more unusual in 5% of DS cases with a trisomy 21 mosaicism (11).

In DS, the PAPP-A level is usually lower and the ß-hCG level higher than in pregnancies with normal chromosomes (19). The median NT (MoM) in DS pregnancies is greater than that in unaffected pregnancies, ranging between 3.93 at 10 gws to 1.55 at 13 gws (20).

2.2.2 Trisomies 18 and 13

The most common trisomies after trisomy 21 are trisomy 18 and 13, the former being more common than the latter (11). The prevalence of Edwards syndrome, trisomy 18, in Finland is between 1/5,000 and 1/8,000 newborns and has been increasing due to increased maternal age (11,21). Because trisomy 18 is associated with a high risk of fetal loss and most pregnancies with it are terminated, the overall prevalence is higher than the prevalence in newborns (21). Only 5–10% of neonates born with trisomy 18 make it past one year (11,21). After the diagnosis of Edwards syndrome, 86% of mothers decide to terminate the pregnancy (21).

The prevalence of trisomy 13, Patau syndrome, in newborns in Finland is lower than that of trisomy 18, 1/10,000. Most trisomy 13 pregnancies (95%) result in fetal loss during early pregnancy or a stillborn child. The life expectancy in trisomy 13 is low and most neonates pass away soon after birth (1,11).

Both Edwards and Patau syndromes cause disturbances in fetal development (11) and can be associated with major structural anomalies. The production of placental hormones is also decreased and both PAPP-A and ß-hCG levels are lower than in normal pregnancies (19).

2.3 Screening terminology

The sensitivity and specificity used in screening tests determine the number of positive and negative results. In DS, a positive result means that the risk for the chromosomal abnormality is increased and the chance that the pregnancy is affected with DS is ≥ 1/250, whereas in the case of a negative result, the chance is < 1/250. Sensitivity describes the test's ability to find cases with the screened condition and specificity describes the test's ability to find cases without it (Figure 3). By increasing the sensitivity of the test, one decreases the specificity. This leads to more positive screening results, including both true (TPR) and false positive results (FPR) (10). Sensitivity and specificity also impact the positive (PPV) and negative

predictive values (NPV). The PPV represents the number of true positives among the positive screening results and the NPV the number of true negatives among the negative results (11).

The sensitivity and specificity of FTS are calculated in a way which gives 5% of the screened population a positive result. This helps to minimize the FPRs (5). The ones with an FPR go through further testing, some of this invasive. This leads to anxiety and additional costs and can result in a miscarriage (11).

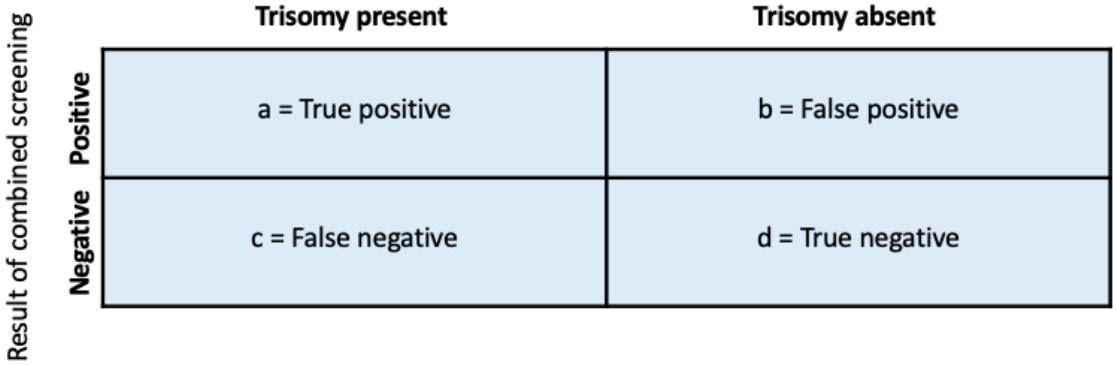

Figure 3: The characteristics of screening. *The sensitivity* is calculated as a/(a+c), *the specificity* as d(b+d) and *the positive predictive value* as a/(a+b).

2.3 False negatives in first trimester prenatal screening

There has been little research on false negative results (FNRs) in FTS, but cases of FNR indicate the same characteristics (22,23). Typically, the cases of FNR in FTS indicate a higher level of PAPP-A, less NT and a lower maternal age compared with the cases of TPR (24). The difference in hCG level has been insignificant in one study and significantly lower in another one done in Finland (23,24). In pregnancies with FNRs, CRL has been demonstrated to be greater than in those with TPRs. Maternal smoking status, ethnicity and conception method might also differ between the cases of TPRs and FNRs. Maternal weight and gestational age at the time of blood tests have not been demonstrated to exhibit significant differences when pregnancies with an FNR are compared with those with a TPR, although the DR is higher when the blood test is taken before 10 gws (22,25).

According to Tanja Schlaikjær Hartwig et al., a little less than half of the cases with FNRs have had a risk between 1:300 and 1:1000 (22). Maternal age affects the DR of trisomies – the number of FNRs is 29% among women between 20 and 25 years and only 5% among women aged 36 or older (24). Also, the median for maternal age is lower among FNRs, being 30.6 and 36 years (22).

When using logistic regression analysis, the NT result has been demonstrated to be the strongest explanatory variable associated with FNRs, accounting for 37.2%. Serum levels of fβ-hCG and PAPP-A account for 30.4% and the remaining factors, accounting for 16.1%, are unknown. The differences between cases of TPRs and FNRs for all of these markers are significant (23). This demonstrates that PAPP-A, fβ-hCG and NT are useful markers in screening for trisomies, but there is room for improvement. Since 16.1% of the remaining factors are unknown, further studies on the subject are necessary.

3 Methology and data collection

3.1 Research framework

This retrospective study analyzed pregnancies with FNRs in FTS during 2014–2016 by comparing their features to pregnancies with TPRs. This study concentrates on pregnancies with postnatally diagnosed chromosomal abnormalities.

All pregnancies were screened at Helsinki University Hospital or Helsinki University district hospitals. As part of the prenatal screening program, FTS was offered free of charge during the first appointment at the local antenatal care clinic. Women were informed about the screening and their consent was obtained.

3.2 Screening protocol

First trimester combined screening was performed according to the guidelines of the Finnish Ministry of Social Affairs and Health (26). The blood samples were collected at 9+0–11+6 gws and the USs were performed at 11+0–13+6 gws, mostly by a trained sonographer, or in some

cases by a specialist in obstetrics or perinatology. The last menstrual period was used to determine the gestational age, but in cases of a discrepancy of more than four days, the CRL of the fetus was used. The NT was measured according to the Fetal Medicine Foundation protocol.

After the determination of the gestational age, the hCG and PAPP-A levels of the previously taken serum samples were expressed in gestational age-specific MoM. Corrections according to maternal weight, ethnicity, diabetes and smoking status were made and the DS risk assessment was made using the LifeCycle database (Perkin Elmer).

An NT of at least 3 mm or cut-off levels of 1/250 for DS and 1/150 for trisomy 18 were used to classify a positive FTS result. Genetic counselling and chromosomal testing were offered to all patients with a positive result.

3.3 Data collection

The data concerning FTS were collected from the hospital database: the data concerning the US screening from Obstetrix and the serum screening data from Weblab. The collected variables were maternal age, gestational ages at blood test and US, fetal CRL (in millimeters) and NT (in millimeters), as well as levels of maternal serum PAPP-A and hCG. Maternal smoking status and weight were unavailable for some of the pregnancies, which made the screening data unusable for this study.

3.4 Statistical analysis

The statistical software program IBM SPSS Statistics 24 (USA) was used for the statistical analysis. A p-value of $< 0.05\%$ was considered statistically significant. Categorical variables were analyzed using the chi-squared test and continuous variables were analyzed with the Mann-Whitney U test. The Kruskal-Wallis test was used when comparing more than two samples. The Bonferroni correction was used for adjusting the significance values. A forward binary logistic regression analysis was used to determine the effect of the variables on FNs.

4 Results

During the study period, around 9,900 screenings per year were performed at Helsinki Women's Hospital and Kätilöopisto Hospital.

4.1 Basic characteristics of prenatally diagnosed trisomies 21, 18 and 13

Between 2014 and 2016, the number of prenatally diagnosed trisomies was more than 200, including 145, 55 and 14 cases of trisomies 21, 18 and 13, respectively. The 124/145 (85.5%) cases with trisomy 21 were detected before 13+6 gws.

The mean maternal age was lowest in cases with trisomy 18 and highest in pregnancies with trisomy 13. The highest mean values for CRL, hCG MoM and gestational age at both blood sampling and US were found in the trisomy 21 group. The highest means for NT and PAPP-A MoM were detected in the trisomy 18 group.

The risk figures for trisomy 21 ranged from 1:5 to 1:4214, with a mean value of 1:156. For Edwards syndrome, the respective risk figures were 1:5 to 1:100000, and a mean value of 1:5460.

The Kruskal-Wallis test did indicate a significant difference for maternal age ($p = 0.825$) and PAPP-A MoM ($p = 0.254$) across the three groups. It indicated significant differences for trisomy 21 and 18 in CRL ($p < 0.001$), gestational age at the time of the USs ($p = 0.001$), gestational age at the time of blood sampling ($p < 0.001$), NT ($p = 0.015$) and hCG MoM ($p < 0.001$) A significant difference was also found for hCG MoM between trisomies 21 and 13 ($p < 0.001$). The descriptive data for these prenatally diagnosed trisomies are presented in Table 1.

Table 1: Basic characteristics of prenatally diagnosed trisomies 21, 18 and 13.

	Trisomy 21 (n=145)	Trisomy 18 (n=55)	Trisomy 13 (n=14)	*p*-value
Maternal age	36.1 ± 5.3	35.4 ± 6.3	36.7 ± 3.4	0.825
Gestational age (BT)	10.7 ± 0.8	10.1 ± 0.9	10.5 ± 0.9	<0.001
PAPP-A (MoM)	0.38 ± 0.27	0.52 ± 0.50	0.41 ± 0.25	0.254
hCG (MoM)	1.90 ± 1.17	0.34 ± 0.22	0.30 ± 0.12	<0.001
Gestational age (US)	12.4 ± 0.5	12.1 ± 0.8	12.3 ± 0.4	0.001
NT (mm)	3.1 ± 1.9	4.3 ± 2.6	2.9 ± 1.4	0.015
CRL (mm)	59.7 ± 7.8	52.9 ± 8.8	55.5 ± 3.2	<0.001
Risk of trisomy 21 (1:X)	142.66 ± 490.22	2384.5 ± 7472.74	3143.0 ± 8386.70	0.149
Risk of trisomy 18 (1:X)	14458.10 ± 30748.15	5459.83 ± 18676.11	9506.54 ± 27596.53	0.001

Table explanation: Numbers are expressed as mean ± standard deviation. BT = blood tests, PAPP-A = pregnancy-associated plasma protein-A, hCG = human chorionic gonadotropin hormone, MoM = multiples of the median, US = ultrasound, NT = nuchal translucency, CRL = crown-rump length

During the study period, 44/145 (30.3%) pregnancies with a prenatally diagnosed chromosomal abnormality ended in delivery. This included two cases with trisomy 18 and 21 cases with trisomy 21. There were no cases of trisomy 13.

In this study, the focus was on pregnancies with trisomy 21 that had an FNR, since the number of pregnancies with trisomies 18 and 13 was too small for analysis. From the total number of 42 postnatally diagnosed children with DS, 17 pregnancies with trisomy 21 were excluded from the study. The causes for exclusion were incomplete data (five pregnancies), twin pregnancy (one pregnancy), an unknown screening status (two pregnancies) and a pregnancy with no FTS (nine pregnancies). Of the total 42, 15 pregnancies had a TPR, which led to the final study group consisting of 14 pregnancies with an FNR in the FTS (Figure 4).

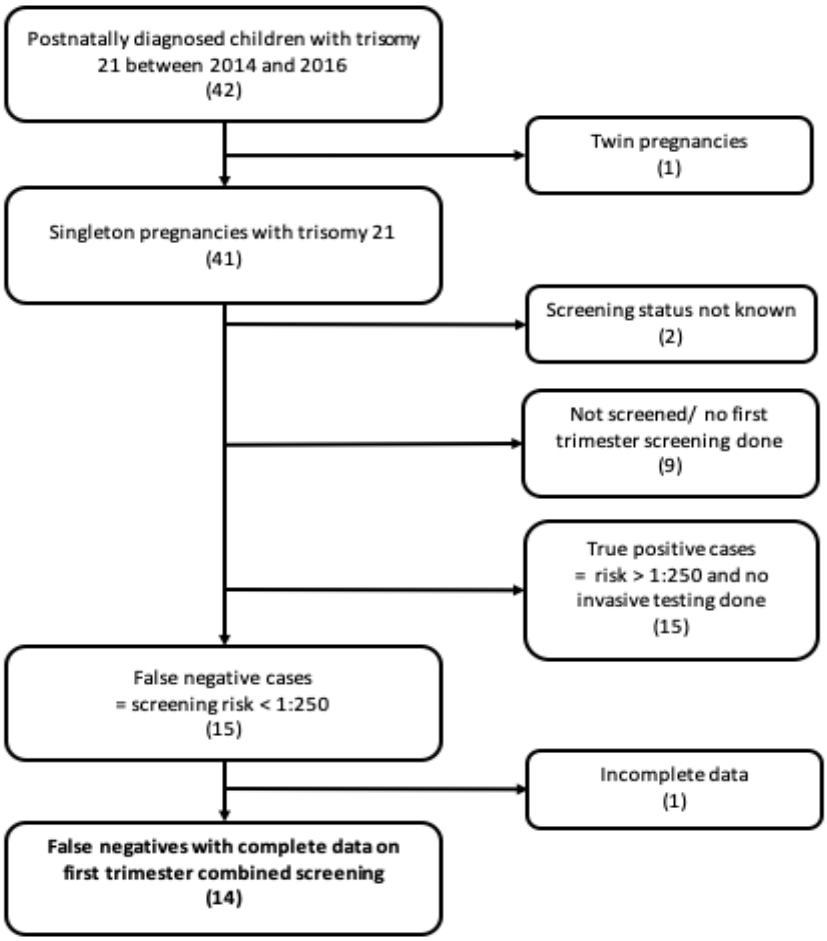

Figure 4: Flowchart demonstrating the study cohort of FTS cases with an FNR for trisomy 21 in Finland between 2014 and 2016.

4.2 Basic characteristics of FNRs

The study group of FNRs consisted of 14 cases. The mean maternal age in the FNR group was 32.0. The means for PAPP-A MoM and hCG MoM were 0.46 and 1.47, respectively. The mean gestational age at the FTS serum sample was 10.7 gws and 12.4 gws at the first trimester US. The means for NT (mm) and the risk figure were 1.24 and 1:1525 (1:295 to 1:5670). The specific parameters of these cases are presented in Table 3.

Table 3: The characteristics of pregnancies with an FNR.

Case	Maternal age	Gestational age (BT)	PAPP-A (MoM)	hCG (MoM)	Gestational age (US)	NT (mm)	Risk of trisomy 21
1	23	10 + 6	0.14	0.42	11 + 4	0.9	1:5670
2	31	11 + 2	0.52	2.23	12 + 4	1.8	1:295
3	37	11 + 4	0.95	2.15	12 + 4	0.8	1:1300
4	39	10 + 0	0.45	1.49	12 + 3	1.1	1:400
5	30	10 + 3	0.26	2.06	12 + 3	1.2	1:390
6	33	10 + 1	0.34	0.75	12 + 3	2	1:320
7	21	10 + 5	0.33	1.07	12 + 1	1.4	1:1900
8	36	10 + 4	0.72	1.73	12 + 3	1.2	1:1684
9	32	10 + 3	0.39	1.39	12 + 4	1.1	1:1501
10	28	10 + 1	0.41	3.05	11 + 6	1	1:928
11	25	11 + 3	0.30	1.46	12 + 4	1	1:1814
12	39	10 + 4	0.48	1.16	12 + 4	1	1:303
13	36	11 + 2	1.00	0.97	12 + 6	1.6	1:4075
14	33	10 + 6	0.14	0.70	12 + 2	1.2	1:768
Mean	32 ± 5.7	10.7 ± 0.5	0.46 ± 0.26	1.47 ± 0.72	12.4 ± 0.3	1.24 ± 0.35	1:1524 ± 1:1567

BT = blood tests, PAPP-A = pregnancy-associated plasma protein-A, hCG = human chorionic gonadotropin hormone, MoM = multiples of the median, US = ultrasound, NT = nuchal translucency

The distribution of risk figures was calculated. Seven cases (50%) had a risk figure between 1:250 and 1:1000 and the majority of these, four cases (28.6%), had a risk figure between 1:250 and 1:500. The distribution of cases with an FNR in relation to the maternal age was calculated by dividing the cases into four age subgroups, 20–24.9, 25–29.9, 30–34.9 and 35–39.9. The vast majority, 70.4%, of all FNs occurred in the age group 30–39.9 and only 28.6% occurred in the group 20–29.9. This is illustrated in Figure 5.

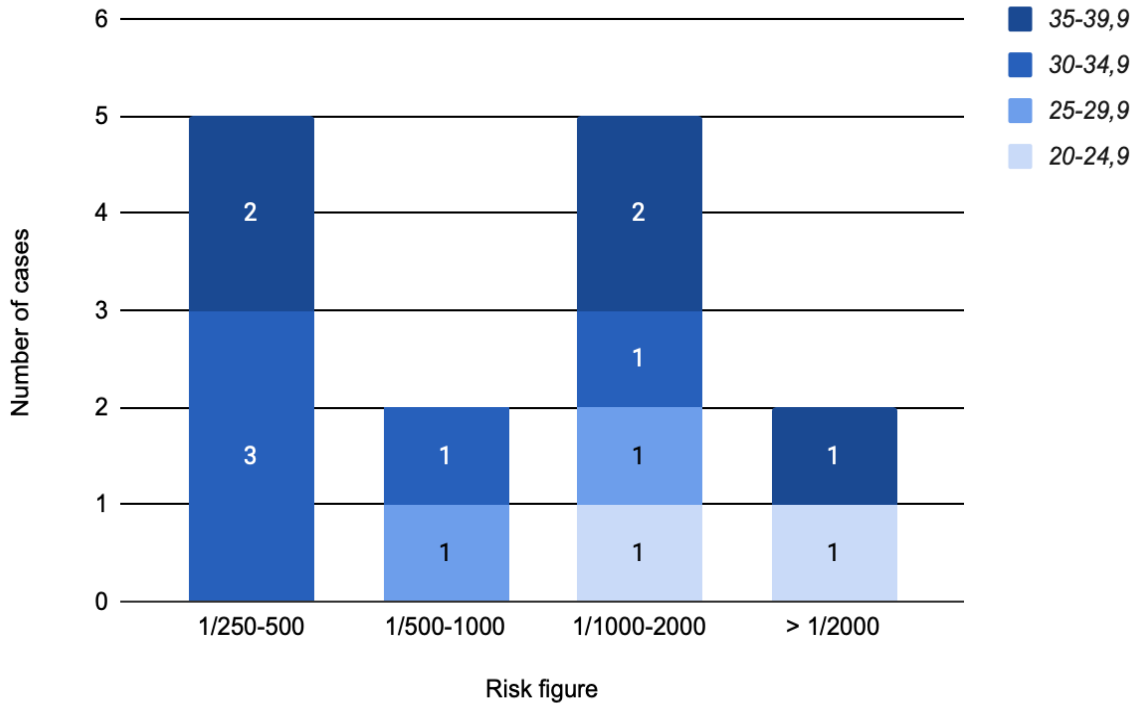

Figure 5: Distribution of risk figures of cases with FNRs among age groups. FNR = false negative result

4.3 Comparison of false negatives and true positives

The cases with FNRs were compared to 119 cases with TPRs and the complete data on FTS. The mean maternal age in cases with FNRs was significantly lower than in cases with TPRs ($p = 0.006$), but there was no difference in the PAPP-A-MoM ($p = 0.114$) and hCG-MoM levels ($p = 0.296$) or in the gestational age at the blood testing ($p = 0.547$ or at the US ($p = 0.788$). The fetal NT was significantly less ($p < 0.001$) among cases with an FNR. The results, with means, standard deviations and *p*-values, are presented in Table 4.

Table 4: Comparison of maternal and fetal variables in false negative and true positive cases using the Mann-Whitney *U* test. The results are presented in means.

Variable	FN cases (n = 14)	TP cases (n = 119)	*p*-value
Maternal age	31.97 ± 5.68	36.46 ± 4.98	0.006
Gestational age (BT)	10.70 ± 0.50	10.66 ± 0.78	0.547
PAPP-A MoM	0.46 ± 0.26	0.36 ± 0.23	0.114
hCG MoM	1.47 ± 0.72	1.90 ± 1.20	0.296
Gestational age (US)	12.40 ± 0.33	12.41 ± 0.52	0.788
NT	1.24 ± 0.35	3.19 ± 1.86	< 0.001

FN = false negative, TP = true positive, BT = blood tests, PAPP-A = pregnancy-associated plasma protein-A, hCG = human chorionic gonadotropin hormone, MoM = multiples of the median, US = ultrasound, NT = nuchal translucency

The distribution of timing in the US and BT was similar among cases with an FNR and a TPR, as presented in Figure 6.

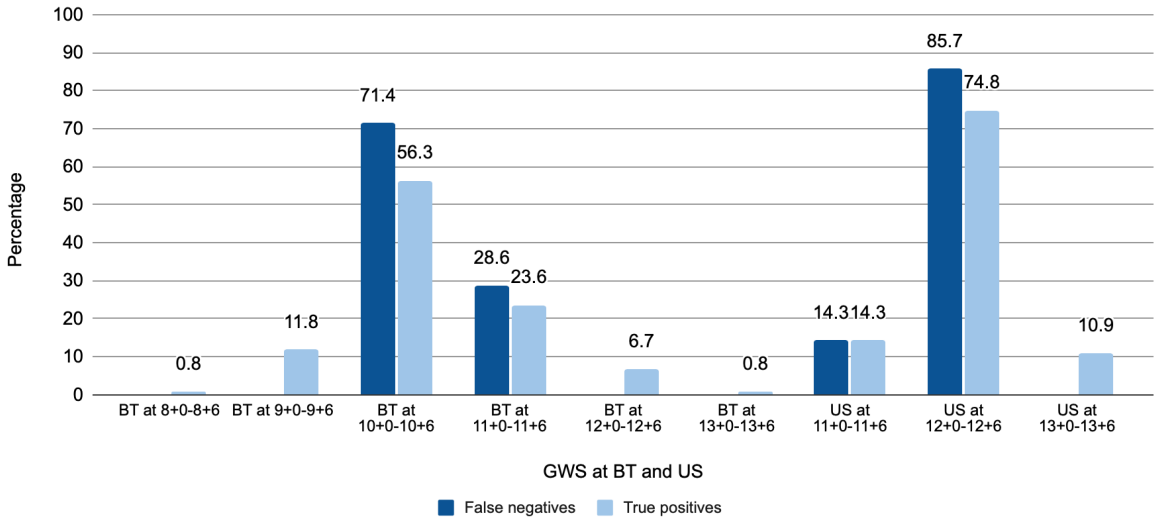

Figure 6: The timing of FTS blood testing and ultrasound. BT = blood tests, US = ultrasound, FN = false negative, TP = true positive

4.4 Predictors of false negatives

A forward binary logistic regression analysis was used to determine which variables accounted for the largest percentage of FNRs and which ones did not have a statistically significant effect. Variables were tested in order of significance.

The variables with a statistical significance between FNRs and TPRs were NT and maternal age. In the regression model, even PAPP-A MoM and hCG MoM increased the explanatory power of the model. These variables account for 85.8% of FNs; the remaining 14.2% is due to factors not studied in this research. The model used had a high specificity for FNs, with its predictive value for them being 85.7%.

The NT had the most powerful discriminating effect ($p < 0.009$), explaining 47.6% of the FNRs. PAPP-A MoM had the second strongest impact and explains a further 15.2% ($p = 0.018$) of cases with FNRs. The differences in maternal age and hCG MoM explain an additional 11.9% ($p = 0.011$) and 11.1% ($p = 0.042$) of cases, respectively. Variations in gestational age at US and blood sampling did not significantly enhance the explanatory properties of this model ($p = 0.201$ and $p = 0.543$, respectively), so they were excluded from the final model.

Table 5: Variables best distinguishing false negatives from true positives, presented with *p*-values for each variable and cumulative Nagerkerke R^2.

Variable	*p*-value	Cumulative R^2 (%)
NT	0.009	47.6
Maternal age	0.018	62.8
PAPP-A MoM	0.011	74.7
hCG MoM	0.042	85.8

NT = nuchal translucency, PAPP-A = pregnancy-associated plasma protein-A, hCG = human chorionic gonadotropin hormone, MoM = multiples of the median

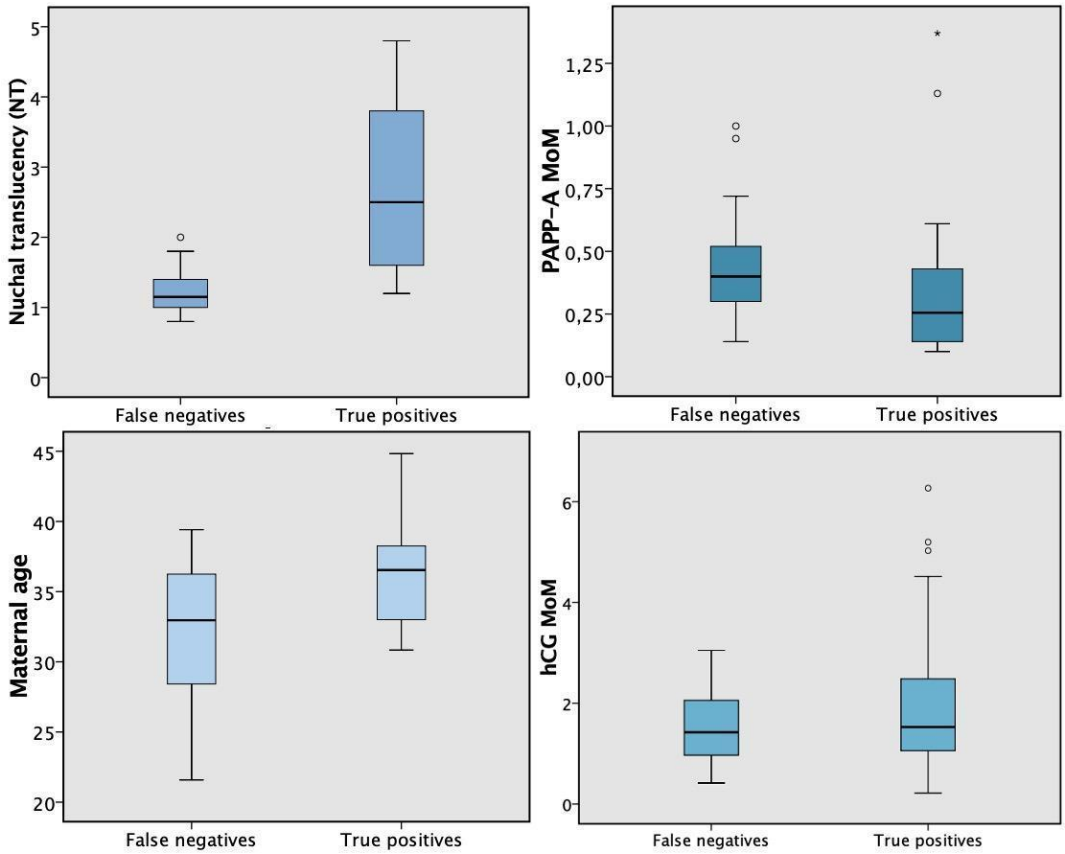

Figure 7: Distribution of data and variability across the discriminating factors: nuchal translucency, maternal age, PAPP-A MoM and hCG MoM.
PAPP-A = pregnancy-associated plasma protein-A, hCG = human chorionic gonadotropin hormone, MoM = multiples of the median

5 Discussion

The sensitivity of screening for chromosomal abnormalities has increased remarkably since its introduction to the Finnish healthcare system. In the late 1970s, when this screening began, the sensitivity and the partition rates were lower (27). The role of research has been essential in increasing the sensitivity and improving the screening methods used. At first only invasive methods, such as amniocentesis and CVS, were used for screening. The application of BTs and risk figures were introduced in the 1990s. Subsequently, new biochemical markers have been discovered and screening methods have been developed. These improvements have made a difference in the number of miscarriages due to invasive screening methods and to increased reproductive autonomy and have made it easier to prepare for the delivery of affected fetuses (1,28).

In this study, we investigated which factors distinguish cases with FNRs from those with TPRs. We found that NT is smaller and maternal age lower among cases with FNRs. This is in line with previous studies on the subject (23,24). Using a logistic regression model, NT appeared to be the most powerful discriminating factor, accounting for 47.6% of cases with FNRs. This was also seen in the study by Marttala et al. (23). Usually, fetuses with DS have an increased NT and an NT of > 3.5 mm has been demonstrated to correlate with the most usual adverse outcomes (29). Even a variation of just 0.1 mm can result in more cases with FNRs and an error of -0.2 mm to +0.4 mm can lead to decreased detection of severely ill fetuses (30). Since there are many factors, such as poor visibility, non-optimal fetal positioning, the US equipment used and the experience of the practitioner, that can affect the measurement, variations in the NT measurement are common (9,30). It has been demonstrated that even a 0.4 mm variation in NT can result in a sixfold difference in the risk figure (9).

We also found that PAPP-A MoM, maternal age and hCG MoM were statistically significant in explaining the FN cases when using the logistic regression model. Similar results were observed in a previous study on the subject by Marttala (23).

However, in our study 14.2% of discrimination is due to unknown factors. Since our study has the same characteristics and used the same FTS DS risk assessment program (LifeCycle) as the study conducted by Marttala et al. (23), our results can be interpreted as being consistent with theirs.

We did not find a statistically significant difference in gestational age at blood tests or US, which is in line with previous findings (23,24).

We found that the majority (70.4%) of cases with FNRs occurred in the age group 30–40 years. This is inconsistent with the findings of a previous study by Schlaikjær Simonsen et al., which found only 17% of cases in this age group (24). This discrepancy could be caused by a possible difference in age correction in the screening software used (22). As in the study by Schlaikjær Simonsen et al., around half of the cases with an FNR had a risk figure between 1:250 and 1:1000. These results speak for the arrangement of contingent screening to decrease the number of FNs. The participants would be divided into three groups based on the risk figure: high risk, intermediate risk and low risk (31). Diagnostic tests would directly be offered only to those in

the high-risk group, whereas those in the intermediate-risk group would continue to normal screening in the second trimester and those in the low-risk group would not undergo further testing. The objective of this method is to reduce unnecessary second trimester screening while still maintaining a high detection rate (32).

Because of the low percentage of women with sufficient available data on smoking status, maternal weight and CRL, we had to exclude these variables from the final analysis. These exclusions most likely limited the explanatory effect of our model. Increasing maternal weight correlates with decreasing levels of PAPP-A MoM and beta hCG MoM, which makes it a possible explanatory factor in FNRs (13). Maternal overweight and obesity have also been demonstrated to increase the failure rate of NT measurements and the time needed to obtain NT measurements, since overweight makes the visualization of fetal structures during the US more difficult (33). A previous study has indicated that FN rates are greatly affected by even slight deviations in NT measurements (30). Nevertheless, the current weight adjustment of serum values seems sufficient, as maternal weight has not been found to be a statistically significant distinction between cases of FNRs and TPRs (22,24). Despite this, having sufficient data on maternal weight to analyze its effect on FNRs could have supported the findings of previous studies. There has been little research on the effect of maternal weight on FNs, so the subject needs to be studied further.

To the best of our knowledge, the difference in smoking status between cases with FNRs and TPRs has not been studied to date, although Spencer et al. have reported that the classification of smoking in terms of yes/no may be too simplified (34). Since smoking has an effect on biochemical markers, decreasing PAPP-A MoM and free beta hCG MoM, it might be a contributing factor in FNRs (13). Had the data been more extensive, the inclusion of a more complex description of smoking status could have provided additional information on the cause of FNRs in FTS.

Since we did not study all the variables, such as ethnicity and fertility method, taken into consideration when calculating the risks for trisomies and since 14.2% of the discriminating factors between cases with FNRs and TPRs remained unknown to us, further research needs to be done.

It has been suggested in the literature that biochemical markers, differences in the organization of the screening program, the level of experience and training of the health care professionals participating in the screening procedure, and the handling of collected samples can explain a number of cases with FNRs (22,35). The underestimation of NT has been demonstrated to be a common issue among sonographers, and it might lead to a decreased abnormality detection rate in FTS. In a study by Torrent et al., sonographers with more years of experience and performing more than 230 US scans per year, as well as those who had completed the Fetal Medicine Foundation (FMF) online course and were regularly audited, were more likely to deliver higher-quality measurements. Feedback has also been demonstrated to increase the number of satisfactory images among low-score sonographers by 48% in a study by Chalouhi et al. (36). However, the screening for chromosomal abnormalities in Finland is arranged according to national guidelines, which decreases the possibility of FNRs due to variations in screening.

A small number of FNs could even be explained by fetoplacental mosaicism, which entails that the genetic abnormality is not present in all cells. Mosaicism explains 1/135 of FNs for trisomy 21 (37). In our study group of 14 cases with an FNR, there were no cases with fetoplacental mosaicism. Therefore, the unknown factors in our study are due to other variables.

The optimization of the time of screening and risk calculation programs used help to guarantee correct results for as many as possible. False positive rates and FNRs lead to psychologically and socially adverse effects among screenees and their families and an increased need for support (1,11). Research has indicated that mothers with FNRs present with higher levels of stress and more negativity towards their children than those who were not offered a test. They were also more likely to blame other people for the FNR than parents who declined a screening test. Despite an FNR, these parents eventually adjusted to having a child affected with DS (38).

Improving the specificity and sensitivity of FTS also has a beneficial effect on the economic aspect of screening. At present, the screening method with the highest specificity and sensitivity is the NIPT (29). Although it is an effective way of detecting DS, its universal use is not yet cost-effective (29,39). However, due to an increasing number of companies offering the NIPT, it is expected that the unit cost will decrease enough in the future to make it cost-effective (39). By improving the detection rate of combined screening, a cost-effective and reliable screening method can be provided. Even if the NIPT were introduced nationwide, US

would still be a necessary tool to estimate the age of the fetus, confirm its viability and provide additional information on other possible structural abnormalities. And even though the risks of the NIPT are lower than those of CVS or amniocentesis, it is still a screening test and not a diagnostic one (5).

Since the risk calculation program requires information on maternal age, duration of pregnancy, the last menstruation, maternal weight, diabetes and smoking status for the calculation of the risk figure (1), a fascinating approach for prospective research could be investigating the combined discriminating effect of these markers between cases with FNRs and TPRs.

Another interesting subject would be investigating whether there is a statistically significant difference across groups in terms of ethnicity and fertility method between FNRs and TPRs, since the effect of these factors on biochemical markers has been demonstrated to be greater than previously presumed (22). As suggested in a previous study by Schlaikjær Hartwig et al., secondary sonographic markers, such as nasal bone, ductus venosus flow and tricuspid regurgitation, could be of interest to study in the characterization of FNRs (22). Even the effect of other biochemical markers on FNRs, such as placental growth factor (PlGF) and plasma C1-protease inhibitor, could be the subject of further investigation (22). Heywood et al. already discovered one new biomarker that could be used in FTS, alongside PAPP-A, hCG and NT, to increase the performance of combined screening. They demonstrated that the levels of plasma C1-protease inhibitor increase in the first trimester in trisomy 21 pregnancies. Nevertheless, the reason for the elevation remains unknown and further research needs to be done with larger study groups to support this finding (40).

Despite improvements to date, there is still room for more. Some variables accounting for FNRs, such as maternal age, cannot be influenced, but others can. For instance, during the measurement of NT there are multiple stages in which external factors can affect the final measurement. The variation in education, the number of NT measurements done by an individual and the duration of the appointment are some of these (1). Even though FTS is researched to increase its sensitivity and decrease the number of FNRs, the goal of achieving 100% sensitivity and 0% FNRs is utopian. Although mathematically it is possible for a test to have 100% sensitivity and specificity, this is highly unlikely to occur in reality. Both sensitivity

and specificity are estimates used in statistics, not error-free descriptives of the test's performance.

Although the sensitivity of the NIPT is close to 100% in singleton pregnancies and it is widely used today, it too has its limitations. FNRs can occur due to fetoplacental mosaicism, vanishing twin syndrome or the presence of maternal chromosomal abnormalities. In addition, FPRs also occur, which is why invasive tests are still performed on some with a positive NIPT result (41,42). There has also been concern regarding the possibilities of prenatal fetal DNA testing. If it is possible to analyze the whole fetal genome in the future, we will have to determine what information is clinically significant and what the parents should be informed about (41). For now, the NIPT is not a cost-effective method, which is why not everyone is able to undergo it (42). This advocates for continuing research on FTS and FNRs, until another method is ready to be taken into use nationwide.

6 Conclusion

This study was conducted to find why screening failed in the pregnancies studied by comparing common characteristics in FN cases to those in TP cases. We found that differences in maternal age and NT are significant between FNRs and TPRs. Moreover, we found that NT, maternal age, PAPP-A MoM and hCG MoM explain 85.8% of the discrimination between FN cases and TP cases and that 14.2% is due to unknown variables.

Overall, our findings are for the most part in line with previous studies and thus confirm that there are unknown variables, that account for some of the FNRs. By improving the performance of FTS, one could avoid unnecessary diagnostic tests and the cost caused by these as well as psychological and social adverse effects among the screenees and their families. Because of NT being the most effective discriminating factor, it is essential to focus on its correct measurement.

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